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In re Application of: Myriam GOLEMBO et al.

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For: METHOD AND COMPOSITION FOR  
TREATMENT OF SKELETAL DYSPLASIAS

Attorney Docket No.: 81408-4300

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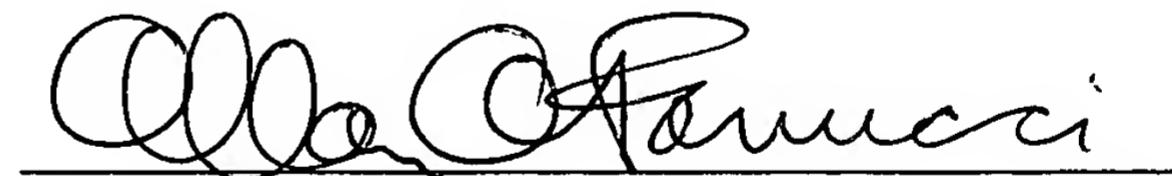
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בקשה לפטנט  
Application For Patent

PROCHON BIOTECH LTD.  
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**METHOD AND COMPOSITION FOR TREATMENT OF SKELETAL DYSPLASIAS**

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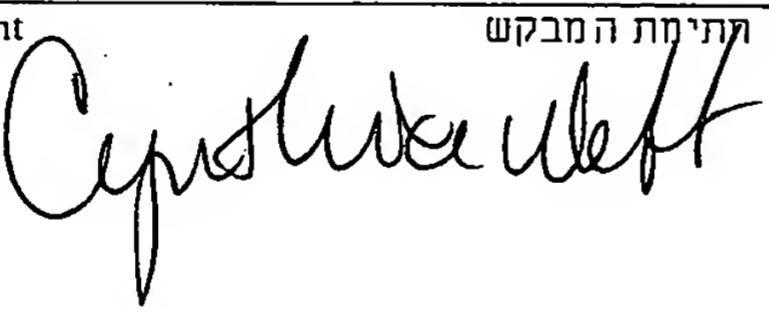
(בעברית) שיטה ותכשיר לטיפול בדיספלזיות של

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ירוי כה: כללי / מוחץ – רצוף זהה / עדיף יוגש to be filed later / attached – P.O.A.: general / individual filed in case הוגש בעניין					
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**PRO/011**

**שיטה ותכשיר לטיפול בדיספלזיה שלד**

**METHOD AND COMPOSITION FOR TREATMENT OF SKELETAL DYSPLASIAS**

**PROCHON BIOTECH LTD.**

**METHOD AND COMPOSITION FOR TREATMENT OF SKELETAL  
DYSPLASIAS**

**FIELD OF THE INVENTION**

5       The present invention relates to treatment of skeletal dysplasias, such as achondroplasia, and in particular to pharmaceutical compositions for bone elongation in abnormal bone growth comprising natriuretic peptides or natriuretic factors.

10      **BACKGROUND OF THE INVENTION**

Endochondral ossification is a fundamental mechanism for bone formation, whereby cartilage is replaced by bone. Endochondral ossification requires the sequential formation and degradation of cartilaginous structures that serve as molds for the developing bones. Longitudinal bone growth is determined 15 by the process of endochondral ossification in the cartilaginous growth plate, which is located at both ends of vertebrae and long bones.

During fetal life and until the end of puberty, longitudinal bone growth takes place via endochondral ossification of the growth plate located at the epiphyses (ends) of long bones. The growth plate is divided into several zones of 20 cartilage forming cells, or chondrocytes, with distinct patterns of gene expression.

In the Reserve Zone, cells are small and relatively inactive. In the adjacent Proliferative Zone, chondrocytes proliferate, arrange themselves in columns and

eventually undergo hypertrophy. In the lower Hypertrophic Region towards the cartilage-bone junction, cells are big and highly active but exhibit no further cell division. The matrix surrounding the hypertrophic cells calcifies and the lowermost cells undergo programmed cell death. Cell death is accompanied by  
5 the removal of the cartilaginous matrix and its replacement by bone through the concerted action of recruited bone cells, namely osteoclasts and osteoblasts.

The process of endochondral ossification is the result of the concerted action of several signaling pathways. The signalling pathway triggered by activation of Fibroblast growth factor (FGF) receptors have been shown to be  
10 involved in several stages of limb and bone development. The FGF receptor 3 (FGFR3) plays a central role in endochondral ossification as demonstrated by the fact that several mutations at various positions in this receptor result in skeletal dysplasias such as Achondroplasia .

Achondroplasia is the most common form of short-limbed dwarfism  
15 occurring with a frequency of 1:20000 live births. Patients show characteristic shortening of proximal long bones (rhizomelia), relative macrocephaly, depressed nasal bridge and lumbar lordosis.

Achondroplasia is mainly caused by a Gly380Arg mutation in the transmembrane domain of the FGFR3 and is transmitted in an autosomal  
20 dominant fashion (Shiang et al. (1994) Cell 78: 335-342 and Rousseau et al. (1994) Nature 371: 252-254). A Gly375Cys mutation has also been reported in some Achondroplasia patients. These mutation affects the process of endochondral

ossification by inhibiting proliferation and delaying maturation of chondrocytes in the growth plate cartilage of long bones, resulting in decreased elongation.

FGFR3 has an inhibitory role in bone elongation as demonstrated by the fact that mice lacking this receptor exhibit a phenotype of skeletal overgrowth.

Natriuretic peptides are known for their role in cardiovascular homeostasis, diuresis, natriuresis and vasodilation. Three isoforms constitute this family: atrial natriuretic peptide (ANP), brain natriuretic peptide (BNP) and C-type natriuretic peptide (CNP). While ANP and BNP are circulating peptides produced by the atria and the ventricle respectively, CNP is hardly found in the circulation and is mainly produced in the brain, in vascular endothelial cells and other tissues where it is supposed to work in an autocrine/paracrine manner (Chen and Burnett, (1998) J.Cardiovasc.Pharmacol. 32 Suppl 3:S22-8).

Natriuretic peptides effect their biological role through two receptors: NPR-A and NPR-B. These receptors have cytoplasmic guanylyl cyclase domains, which are activated upon ligand binding and lead to accumulation of intracellular cGMP. Some of the effects of cGMP are mediated through two known protein kinases: cGMP-dependent protein kinase I and II. The peptides bind the receptors with different affinities:  $\text{ANP} > \text{BNP} >> \text{CNP}$  for NPR-A and  $\text{CNP} > \text{ANP} \geq \text{BNP}$  for NPR-B. The tissue distribution of each receptor is different. While NPR-A is expressed in vasculature, kidney and adrenal glands, NPR-B is mainly expressed in the brain.

A third receptor, NPR-C has no cytoplasmic guanylyl cyclase domain and is generally considered to be a clearance receptor for removing natriuretic

peptides from the circulation, though some other biological functions have been attributed to it (Murthy and Makhlof. (1999) JBC 274:17587-17592). This is the most widely distributed receptor and is expressed in almost all the tissues that express a guanylyl cyclase receptor.

5 Natriuretic peptides are further cleared from the circulation by degradation. The peptides are cleaved at specific sites, by the neutral endopeptidase 24.11 (NEP) which is found in endothelial cells covering the vascular walls. BNP is more resistant to this degradation while ANP and CNP are readily degraded by this enzyme. Inhibition of the endopeptidase by inhibitors  
10 including for instance the compounds thiorphan or candoxatril (Ohbayashi et al. (1997) Clin. Exp. Pharmacol. Physiol. 25: 986-91; Brandt et al. (1997) Hypertension 30: 184-90), increases the concentration of the endogenous or administered peptides in the circulation.

Transgenic mice, over-expressing BNP show skeletal phenotype  
15 characterized by overgrowth of the axial and appendicular skeleton (Suda et al. (1998) PNAS 95: 2337-2342). Moreover, mice that are null mutants for the clearance receptor, NPR-C, exhibit a similar skeletal overgrowth, consistent with a role for the local modulation of natriuretic peptides levels by NPR-C (Matsukawa et al. (1999) PNAS 96: 7403-7408 ). CNP and its specific receptor,  
20 NPR-B have been shown to be expressed in the proliferating zone of the growth plate in fetal mouse tibia while NPR-C has been shown to be expressed in the region of hypertrophic chondrocytes and in osteoblasts (Yamashita et al. (2000) J.Biochem 127: 177-179).

Furthermore, *ex vivo* experiments with fetal bone organ culture from wild type animals have shown that CNP, more than BNP and ANP, can induce bone elongation (Yasoda et al. (1998) J. Biol. Chem. 273: 11695-11700, Mericq et al. (2000) Pediatric Research 47: 189-193).

5 While much is known about the components of signaling pathways that contribute to the process of endochondral ossification, little is known about the complex interactions between them that coordinate longitudinal bone growth.

Nowhere in the background art is it taught or suggested that natriuretic peptides may be useful for the treatment of skeletal dysplasias of any type, nor of  
10 achondroplasia in particular.

## SUMMARY OF THE INVENTION

The present invention provides a method and composition for treating  
5 skeletal dysplasias such as achondroplasia. The method and composition of the  
invention effect bone elongation, inter alia, by increasing the size of the  
hypertrophic zone of the bone, specifically of limb bones.

The invention provides the use of natriuretic peptides (NP) in effecting  
bone elongation and treating skeletal dysplasias.

10 The term "natriuretic peptides" or "NP" as referred to herein relates to  
any of the three isoforms, atrial natriuretic peptide (ANP), brain natriuretic  
peptide (BNP) and C-type natriuretic peptide (CNP) and to any functional  
derivatives thereof.

The method of the invention for treating skeletal dysplasias includes the  
15 step of administering to a patient an effective amount of an NP. In one  
embodiment of the invention the natriuretic peptide is CNP. The method may  
further include a step of administering to the patient an inhibitor of neutral  
endopeptidase 24.11 (NEP). Suitable compounds for inhibiting NEP are known in  
the art, including but not limited to thiorphan or candoxatril. Administration of  
20 such an inhibitor of neutral endopeptidase may be performed either separately or  
simultaneously with the administration of NP.

It may also include administering an inhibitor of the clearance receptor  
NPR-C either alone or in conjunction with administration of NP.

Administration of NP to a patient can be achieved by any suitable route of administration, including but not limited to injecting NP to the patient, inhalation, or implantation of a depot into the patient. NP may further be administered by an osmotic pump, such as an Alzet pump. The osmotic pump can 5 be implanted subcutaneously, or at any other appropriate site. Preferred sites may be close to the target site of action namely in proximity to the long bones of the limbs.

A further method of administration may be implantation of NP secreting cells. Methods for implantation of NP secreting cells include encapsulation of the 10 cells in any immunologically inert matrix. One non-limitative example of such a matrix is an alginate-polylysine-alginate (APA) complex encapsulating the cells. These methods of administration and other known methods may be utilized alone or in combination for treating skeletal dysplasia.

The present invention further provides a composition for treatment of 15 skeletal dysplasias, such as achondroplasia. The composition includes an NP and any pharmaceutically acceptable diluent or carrier thereof. According to one currently more preferred embodiment of the invention the natriuretic peptide is BNP. According to one currently most preferred embodiment of the invention the natriuretic peptide is CNP. The composition may further include any substance, 20 molecule or vehicle capable of increasing the NP stability in vivo. For example the composition may include an inhibitor of neutral endopeptidase 24.11 (NEP), including but not limited to thiorphane or candoxatril.

The composition may be in any form suitable for being administered to a patient, including but not limited to in liquid form or in suspension.

Also provided is a composition for treatment of skeletal dysplasias that includes NP secreting cells encapsulated within an immunologically inert matrix.

- 5 One currently preferred embodiment includes NP secreting cells encapsulated in an alginate-polylysine-alginate (APA) complex and a suitable carrier thereof.

## BRIEF DESCRIPTION OF THE DRAWINGS

The present invention will be understood and appreciated more fully from the following detailed description taken in conjunction with the drawings in  
5 which:

Figure 1 shows the growth rate curves for the different animals in the ex vivo experiment; and

Figure 2 shows the interpolation of the growth rate curves of Fig. 1.

## **DETAILED DESCRIPTION OF THE INVENTION**

Achondroplasia is characterized, inter alia, by shortening of proximal long bones. In humans the bone growth plate is active until puberty and bone growth is thus achieved until puberty. Thus, treatment aimed at bone elongation, 5 for example, by increasing the size of limb bone hypertrophic zone, would be advantageous during this period.

Treatment of skeletal dysplasias such as achondroplasia, includes treating a shortened bone with NP. The bone may be treated by administering to a patient an effective amount of NP. The amount of the active ingredient administered will 10 be determined by the attending physician and is generally proportional to the patient's weight

According to the present invention it is now disclosed that NP can induce bone elongation in situation of abnormal bone growth such as those typical of skeletal dysplasias.

15 The role and use of NPs in bone elongation in situations of abnormal bone growth and in the treatment of skeletal dysplasias, such as achondroplasia, is demonstrated in the following examples and experiments.

### **Ex Vivo Experiment**

Femurs derived from a mouse Achondroplasia model (Ach369 knock-in) 20 were dissected from P0 wild type, heterozygote and homozygote littermates and cultured in the presence of natriuretic peptides for 24 days.

### **Protocol for bone culture**

Femora are derived from Ach 369 homozygote, heterozygote or wt littermates. (Ach369 mice are knock-in mice carrying the mutation for achondroplasia Gly→Cys at position 369 homologous for the human position 375).

5 Two femora from newborn animals, aged P0, are dissected and cleaned from surrounding muscle.

The femora are incubated (one/well) in serum-free media consisting of :

MEM ALPHA medium (without ribonucleosides and deoxyribonucleasides, GIBCO BRL);

10 0.2% BSA (fraction V, Sigma);

Penicillin-streptomycin-nyastatin solution (Beit Haemek, Israel); and

1mM b-glycerolphosphate disodium.

Femora length was measured using an eyepiece micrometer in a dissecting microscope.

15 CNP was added to culture medium at indicated concentration.

Medium was changed and femora length measured every 3 days.

## Results

It is known from *ex vivo* experiments with fetal bone organ culture from  
20 wild type (normal) animals that CNP, more than BNP and much more than ANP, can induce bone elongation. According to the present invention it si now shown that natriuretic peptides can induce longitudinal growth of Achondroplasia-derived bones.

CNP increased the total longitudinal growth of femurs derived from Ach369 and wild type mice compared to vehicle treated animals.

Figs. 1 and 2 depict the growth rate curves for the different animals in the experiment. The growth rate curve shows two different stages of growth: an initial stage (days 0-3) in which growth is quick and is most affected by the presence of natriuretic peptides and a second slower linear rate. Interestingly, at this stage, femurs derived from homozygote Ach animals respond better to CNP than femurs derived from heterozygote animals while bones derived from wt animals are very slightly influenced by CNP. This is summarized in Table 1.

10

Genotype	Treated/untreated growth rate
Ach369 homozygote	2.28
Ach369 heterozygote	1.7
Wild type	1.07

This suggests that Achondroplasia derived rudiments have got the capacity to respond to CNP for a longer period of time. Fig. 1 shows that with time, NP treated femurs from Ach homozygote pups become longer than those from untreated Ach heterozygote mice and a plateau has not been reached. In fact, the change in growth rate was more marked in femurs derived from homozygote animals than in those from heterozygote animals and finally in wt animals.

Natriuretic peptides lead to an increase in the size of hypertrophic zone

Most of the elongation induced by natriuretic peptides observed in bone culture results from epiphyseal growth and not from elongation of the mineralized diaphysis. Morphological analysis of the cellular composition of the growth plate in treated samples revealed that there is a relative increase in the size of the proliferative region and a marked increase in the size of the hypertrophic zone.

5 This could be due to an increase in the pool of growth plate chondrocytes which subsequently differentiate into hypertrophic cells, or to "accelerated" maturation which would also lead to an increase in bone length.

BNP specific for mouse was synthesized and labeled with biotin. This  
10 BNP was also tested in bone culture on bones derived from Achondroplasia and it could also induce bone elongation. Nevertheless, BNP was used at a higher concentration than CNP.

Histological analysis of the distribution of biotinylated BNP in the growth plate shows that it is apparent both in proliferative and in hypertrophic  
15 cells. Recent studies have shown the distribution of the NP receptors in the growth plate: NPR-B is expressed in proliferative cells while NPR-C is expressed in hypertrophic cells (Yamashita et al. (2000) J.Biochem 127: 177-179). Therefore, BNP could exert its effect on bone growth either by stimulating the activity of NPR-B or by blocking the clearance of the endogenous peptide, CNP,  
20 through NPR-C.

### In Vivo Experiments

#### **IP administration of drugs to Achondroplasia pups**

The fastest growth period for mice is during the first month of life therefore, by initiating the experiments as early as possible it is expected to influence growth the most. One disadvantage is the fact that at this age, there is a limit on volume delivered. Delivery of drug can not be performed intravenously

5 (IV) or by implantation of a continuous delivery device like a pump.

### Animals

Heterozygote mice for the Achondroplasia mutation, aged P4-P5, weighing approximately 3.5 gr.

10 Animals are randomly separated into groups of 3 animals/group and ear marked. Animals are distributed 6-7 individuals/cage with one foster mother.

### Materials and procedures

Animals are injected daily with drug intraperitoneally (IP) in a volume not exceeding 50 $\mu$ l. This volume can be increased proportionally to weight increase.

15 Animals are weighed and the tail length is measured on day 0 and every 2 days thereafter

The treatment is continued for 2 weeks

At the end of the administration period animals are sacrificed and skeletal elements are measured and analyzed by histology.

20 CNP is administered at a concentration of 10<sup>-7</sup>M and 10<sup>-6</sup>M during 2 weeks. In all experiments in mice CNP was diluted in 1x PBS.

## **Administration of drugs to Achondroplasia model mice by continuous release from Alzet pumps**

Release of drugs by osmotic pumps provides a continuous supply and a constant amount of circulating drug. Furthermore, it enables directed release of  
5 the drug closer to the target site. Nevertheless, this procedure can only be performed in older mice.

### Animals

Heterozygote mice for the Achondroplasia mutation, aged P12-P14,  
weighing approximately 10 gr.

10 Animals are randomly separated into groups of 3 animals/group and ear marked. Animals are distributed 6-7 individuals/cage with one foster mother.

### Materials and Procedure

The Alzet pumps used have a total volume of 100 $\mu$ l and a release constant of .25  $\mu$ l/hour during 14 days.

15 Pumps are filled with appropriate drug and calibrated for 4-6 hours  
Pumps are implanted subcutaneously (SC) on the back of anesthetized mice and the contents are directed with a catheter to the femoral artery of one hindlimb.

Mice are monitored for recovery and returned to mothers.

20 Mice are weighed and measured every 2 days.

At the end of the administration period animals are sacrificed and skeletal elements are measured and analyzed by histology.

This experiment was performed with a concentration of  $10^{-4}$ M of CNP in pump to obtain a concentration of  $10^{-7}$ M in the blood stream. In all experiments in mice CNP was diluted in 1x PBS.

## 5 **Implantation of alginate encapsulated cells that secrete CNP**

Transfected NIH-3T3 fibroblasts with mouse CNP gene were tested for CNP is production and secretion.

The cells were encapsulated in APA (alginate-polylysine-alginate) complex and were implanted intraperitoneally (IP) into P5 mice (Ach369 and wt).

10 Mice are weighed and tail length measured every 2 days for 3 weeks.

## **Administration of drugs together with an NEP inhibitor to Achondroplasia model mice by continuous release from Alzet pumps**

The NP have a short half-life in the circulation, probably due to the activity of the neutral endopeptidase.

15 An experiment is performed in which CNP is administered at  $10^{-4}$ M in pump to obtain a concentration of  $10^{-7}$ M in blood stream together with thiorphan (NEP inhibitor) at a concentration of 10 mg/ml in pump (10  $\mu$ g/ml blood stream) for 2 weeks. Administration is done by using an Alzet pump that releases its contents subcutaneous. The same animals are injected with extra CNP daily, IP, 20 together with 0.1% BSA at a concentration of  $10^{-7}$ M.

A similar experiment is performed with biotin labelled BNP that was administered to animals which also received thiorphan. BNP is at  $10^{-4}$ M in pump

to obtain a concentration of  $10^{-7}M$  in blood stream, and  $10^{-7}M$  is injected daily as for CNP.

In a similar manner, any combination of NPs with compounds that contribute to NP stability in the circulation can be administered to a patient for 5 efficient bone elongation and/or treatment of skeletal dysplasias. Such combinations may include a mixture of CNP and BNP, NPs in combination with peptidase inhibitors, NPs in combination with NPR-C antagonists, and so on.

It will be evident to the skilled artisan that administration of NPs according to the principles of the present invention can be performed by any 10 suitable route of administration, utilizing any suitable pharmaceutically acceptable carrier or diluent. Under certain circumstances specific formulations that enable or enhance targetting of the active principle to the bone or growth plate may be utilized such as but not limited to use of the NPs in combination with vehicles such as liposomes, microemulsions, microcapsules, microspheres, 15 and the like. It is also intended according to the principles of the present invention that molecules may be conjugated to the active principle (NP) to prolong the halflife or in order to enhance targetting. Candidate molecules include Antibody peptidesm hydroxyapatite, glucosamine, collagen especially collagen type X, polyGlu or polyAsp and other molecules having affinity for the growth plate.

20 It will be appreciated that many improvements may be achieved by stabilization of the NP or otherwise achieveing a prolonged half life or improved phamacokinetic profile. For example, functional derivatives of NPs having enhanced stability in vivo, such as peptides having altered sequences or

configurations, may be administered for treatment of skeletal dysplasias and/or bone elongation.

Further, cells can be engineered to produce and secrete functional derivatives of NPs having enhanced stability *in vivo* and these cells may be  
5 encapsulated in APA (alginate-polylysine-alginate) complex and implanted intraperitoneally for treatment of skeletal dysplasias and/or bone elongation.

It will be appreciated by persons skilled in the art that the present invention is not limited to what has been particularly shown and described  
10 hereinabove. Rather the scope of the present invention is defined only by the claims which follow:

## CLAIMS

1. A method for increasing the size of a bone hypertrophic zone in abnormal bone growth.comprising the step of treating the bone with an effective amount of at least one natriuretic peptide, or variants thereof.
- 5       2. The method according to claim 1 wherein the at least one natriuretic peptide is CNP.
3. The method according to claim 1 wherein the at least one natriuretic peptide is BNP.
4. The method according to claim 1 further comprising inhibiting the  
10      natriuretic peptide clearance receptor.
5. The method according to claim 1 wherein the bone is a limb bone.
6. The method according to claim 5 wherein the limb bone is an  
achondroplastic bone.
7. A method for bone elongation in abnormal bone growth, comprising the  
15      step of treating the bone with an effective amount of at least one natriuretic peptide, or variants thereof..
8. The method according to claim 7 wherein the at least one natriuretic peptide is CNP.
9. The method according to claim 7 wherein the at least one natriuretic  
20      peptide is BNP.

10. The method according to claim 7 further comprising inhibiting the natriuretic peptide clearance receptor.

11. The method according to claim 7 wherein the bone is a limb bone.

12. The method according to claim 11 wherein the limb bone is an  
5 achondroplastic bone.

13. Use for the preparation of a medicament for treating skeletal dysplasia of at least one natriuretic peptide.

14. Use according to claim 13 wherein the at least one natriuretic peptide is CNP.

10 15. Use according to claim 13 wherein the at least one natriuretic peptide is BNP.

16. Use according to claim 13 wherein the skeletal dysplasia to be treated comprises achondroplasia.

17. A pharmaceutical composition for bone elongation comprising at least one  
15 natriuretic peptide and a carrier thereof.

18. A pharmaceutical composition according to claim 17 wherein the at least one natriuretic peptide is CNP.

19. A pharmaceutical composition according to claim 17 wherein the at least one natriuretic peptide is BNP.

20. A method for treatment of skeletal dysplasias comprising the step of administering to a patient a therapeutically effective amount of at least one natriuretic peptide.

21. The method according to claim 20 wherein the at least one natriuretic peptide is CNP.  
5

22. The method according to claim 20 wherein the at least one natriuretic peptide is BNP.

23. The method according to claim 20 further comprising the step of administering to the patient an inhibitor of neutral endopeptidase 24.11 .

10 24. The method according to claim 23 wherein the inhibitor of neutral endopeptidase is thiorphan or candoxatril.

25. The method according to claim 23 wherein the step of administering to the patient an inhibitor of neutral endopeptidase is performed simultaneously with the step of administering to a patient an effective amount of at least  
15 one natriuretic peptide.

26. The method according to claim 20 wherein the skeletal dysplasia is achondroplasia.

27. A composition for treatment of skeletal dysplasias comprising at least one natriuretic peptide and a pharmaceutically acceptable carrier or diluent.

20 28. A composition according to claim 27 wherein the at least one natriuretic peptide is CNP.

29. A composition according to claim 27 wherein the at least one natriuretic peptide is BNP.

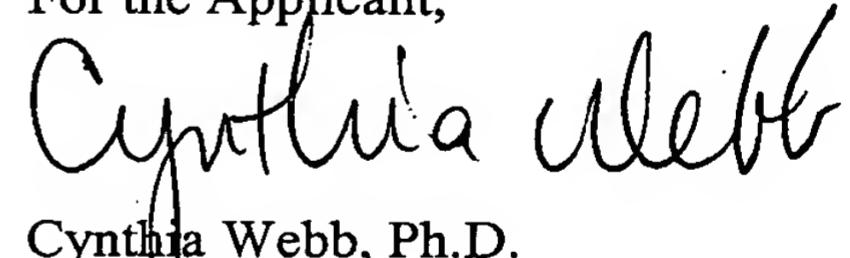
30. A composition according to claim 27 further comprising an inhibitor of neutral endopeptidase.

5 31. A composition for treatment of skeletal dysplasias comprising natriuretic peptide secreting cells encapsulated within an inert matrix.

32. A composition for treatment of skeletal dysplasias comprising natriuretic peptide secreting cells encapsulated within an alginate-polylysine-alginate complex and a carrier thereof.

10

For the Applicant,



Cynthia Webb, Ph.D.

Patent Attorney

Webb, Ben-Ami & Associates

PRO/011

### Abstract

The present invention discloses pharmaceutical compositions for the treatment of skeletal dysplasias, comprising as an active ingredient at least one natriuretic peptide. Unexpectedly, it has been shown that the natriuretic factors may be effective for bone elongation in situations of abnormal bone growth especially for achondroplasia. The effects of the natriuretic peptide may be further enhanced by prolonging its residence time or action at the target site.

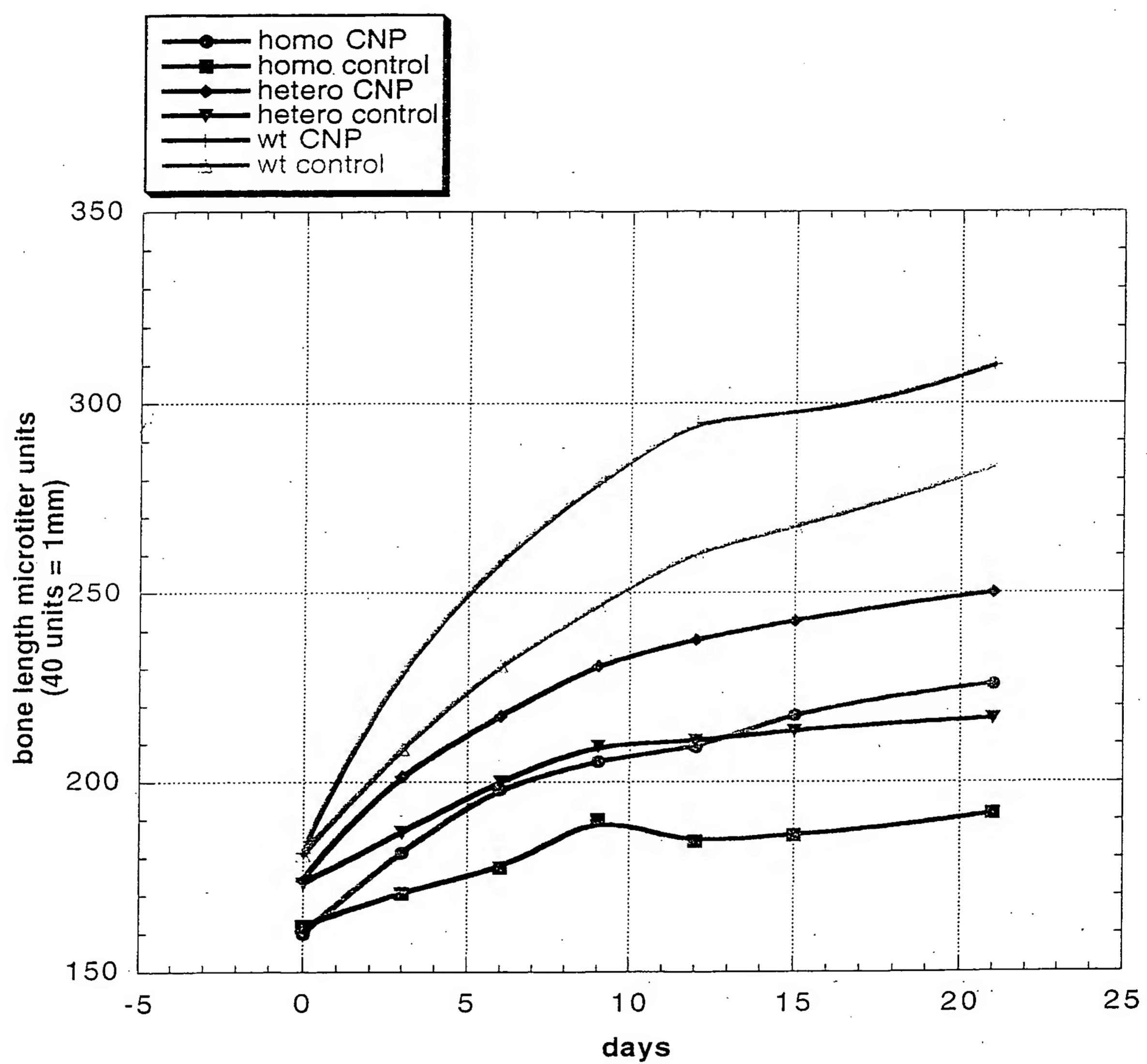
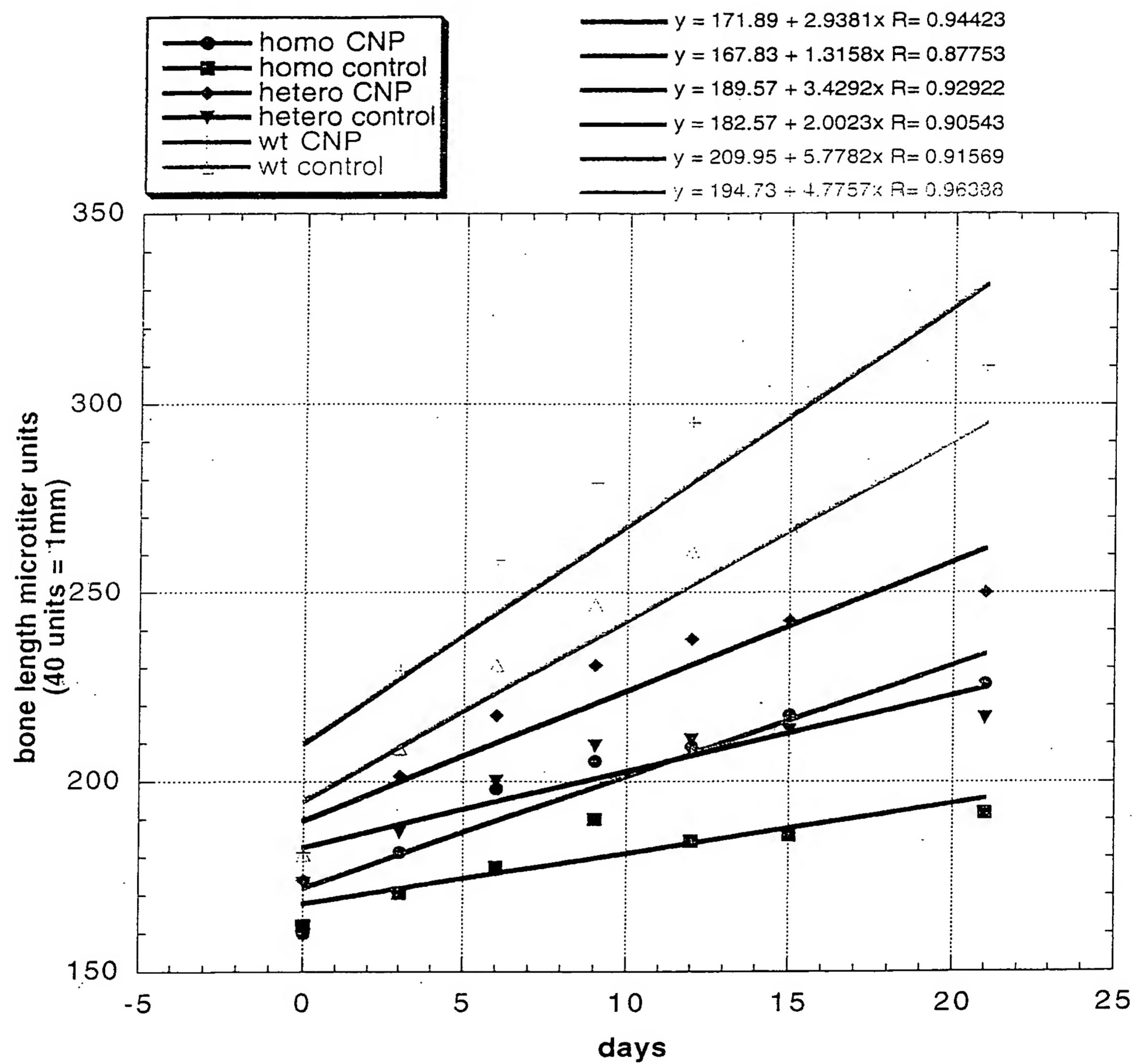


FIGURE 1

**FIGURE 2**

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